

lls ~~IN SITU IDENTIFICATION OF TARGET STRUCTURES E.G. IN VIVO SELECTION METHOD FOR A PHAGE LIBRARY~~

lls The invention relates to a selection method and the products resulting from the method. More specifically the invention relates to a method for
5 acquiring one or more binding structures against a target structure by means of a first library of one or more binding structures linked to genetic and/or other identifying information.

The phage display technology offers a powerful means to select antibody fragments with rare specificities from large libraries (1). Antibody
10 libraries from immune, naive and semisynthetic sources of 10^{10} diversity and beyond (2-4) can efficiently be examined by the antigen driven selection principle. Typically, for pure protein antigens or haptens, affinity chromatography or panning techniques have been used to achieve enrichment of one hundred to ten-thousand fold by each selection round (5-7). Recently, selection systems based on complex solid-phase antigens such as viable bacterial
15 cells (8), human erythrocytes (9) or other cell suspensions (10-13) have been described. These introduce the use of antibody phage selection to "dissect" a cell surface by cloning antibodies specific to individual cell surface antigens. This can be either random or be directed to a phenotypically defined subset of antigens. One example of a situation where the method described could
20 be highly advantageous is research with the aim to identify new target structures for targeted tumor therapy.

The target tumor phenotype for antibody-guided tumor immunotherapy is typically an invasive/metastatic tumor cell population. Such
25 tumor cells could not be expected to be adequately represented by cultured tumor cell lines primarily selected for proliferation in an artificial culture environment. Instead, continued *in vitro* culture would aggravate phenotypic drift and downregulation of expression of tumor-associated molecules that was sustained *in vivo* by growth architecture and by the influence of soluble or mesenchymal tissue constituents (15,16). Selection of antibodies for
30 tumor targeting, being critically dependent on the homogenous and

abundant expression of the target epitope, should preferentially be performed using the authentic tumor cell phenotype during both the antigen driven selection stage and the screening of individual clones.

Thus, successful selection from large libraries of antibody phage specific to known purified antigens has been demonstrated by a number of laboratories. It has also been shown that intact bacterial or mammalian cells, which represent a more complex surface of antigens, can be used for antibody phage selection. However, the use of cell suspensions for phage selection can only generate reagents to cell surface antigens; other components of a tissue such as the extracellular matrix, inflammatory infiltrates, vasculature and components of angiogenesis and intracellular antigens can not be reached by the cell selection approach. In addition, culturing of cells through several passages will introduce selection and phenotypic changes compared to the original cell isolated from a specific tissue environment. Accordingly, there is a demand for a method which could be particularly useful for the identification of antigens which are only expressed *in vivo*. Such a method should overcome a major limitation of currently used selection protocols.

Jul The purpose of the present invention is to provide an extension of the application of phage technology for the selection of antibodies to complex antigens, thus making it generally applicable to identify antibodies directed against a number of important and/or novel target antigens and epitopes which are not accessible in *in vitro* culture systems, which would facilitate identification and dissection of antigens which are exclusively expressed *in vivo*. Of course, the method could generate antibodies to any target structure displayed within the tissue section.

These include antigens whose expression is induced by epithelial cell-mesenchymal cell and cell-matrix interactions, or is tightly regulated spatially or temporally during embryonic development and by a specific patho-physiological process, e.g. tumor progression (14).

A further purpose of the invention is to provide a method with a broad applicability as an analytical tool in studies of cell and tissue develop-

ment and of antigenic phenotypes associated with tissue pathology of various conditions. Hereby is provided a novel and unique means for a much less biased dissection of specificities among a broad range of tissue expressed antigens analogous to the use of naive antibody libraries for selection of reagents to single antigens (23).

Still a further purpose of the invention is to include in the method by means of negative selection a specific removal of undesired antibodies in order to promote the enrichment of phenotype specific specificities.

In order to achieve these purposes the method according to the invention has obtained the characterizing features of claim 1.

The method according to the invention should have a broad applicability in a variety of research fields (e.g. in the study of embryogenesis, angiogenesis and tumor biology) where novel antibody specificities could be used to define temporal or spatial expression of normal or pathologic tissue phenotypes. The phage selection method described below and applied to selection of melanoma reactive scFv antibodies on sections of metastatic melanoma tissue should thus only be considered and construed as a specific example of the present invention. Thus, the usefulness of the method should not be restricted to identification of tumor associated antigens, but rather be generally applicable for the *in situ* identification of target structure expression patterns in both normal and pathological tissues.

In order to further explain the invention reference is made to the accompanying drawings in which

sub C1 FIG 1 is a histogram demonstrating yield (output/input of colony forming units, CFU) of C215 (black bars) and D1.3 (white bars) scFv phage and enrichment of specific phage at various amount of phage added in a volume of 100µl. The ratio, 1:15, of the C215 and D1.3 phage in the dilution series was kept constant.

FIG 2 is a histogram demonstrating correlation of phage yield (A) and specific C215 scFv phage enrichment to the fraction of antigen positive surface area (B) in composite tissue sections.

FIG 3 depicts the yield of phage enrichment after various selection rounds. Diagram (A) shows the effects on phage yield and frequency of C215 scFv producing clones after multiple rounds of tissue-based selection of pre-determined frequencies of 10^{-2} (circles), 10^{-4} (squares) and 10^{-6} (triangles) specific C215 phage in mixtures with unspecific D1.3 phage. Diagram (B) shows the increase of phage yield after multiple rounds of selection of immune primate scFv library and internal reference phage D1.3 (triangles) on melanoma tissue sections. In the third selection round, the predominance of scFv clones directed to epitopes common to melanoma samples of two different patients (circle and square) was demonstrated. Phage was eluted by Ala-64 subtilisin digestion.

FIG 4 is a cytofluorograph depicting specific binding of the K373 scFv-SEA fusion protein (solid line) and no primary antibody control (dotted line) to the FM3 human melanoma cell line (a) and lack of binding to human peripheral blood lymphocytes (b).

FIG 5 is a graph showing the specificity of phage enrichment on tissue sections; and

FIG 6 is a tissue based selection demonstrating the effect of negative selection on reduction on undesired phage antibody specificity.

According to the invention one or several binding structures are obtained against a target structure. Such an acquirement of binding structures comprises identifying, producing, characterizing, selecting, enriching, or defining the structures. Such binding structures can for example be one or several monoclonal antibodies, one or several proteins, one or several peptides, or one or several organochemical entities. The displayed target structure may include one or several previously uncharacterized and/or unpurified and/or unknown molecules.

The method according to the invention comprises the steps of
(a) reacting a first library with the displayed target structure to bind some of the binding structures to the displayed target structure;
(b) separating the displayed target structure and bound binding structures

from unbound binding structures;

(c) recovering bound or unbound binding structures; and

(d) amplifying bound or unbound binding structures to create a second enriched library of binding structures.

5 ~~In order to make the method more effective and specific the steps (a) through (c) as well as the steps (a) through (d) can be repeated. The amplification of bound binding structures can be obtained by means of synthesis in growing bacterial cells, PCR (polymerase chain reaction) synthesis, and chemical synthesis. Thus, monoclonal, single-entity, homogenous, uniform and/or other binding structures can be isolated and/or amplified from the second, or third, or fourth etc enriched library.~~

10 In contrast to established selection methods based on known and purified target structures, the method according to the invention represents a generally applicable tool for the identification of binding structures directed to displayed target structures which are exclusively displayed *in vivo* and/or *in situ*. The displayed target structure is expressed as an authentic phenotypic epitope. The set of displayed target structures can for example be target structures from a whole cell. The displayed target structure can be obtained within a set of displayed target structures representing the authentic *in vivo* and/or *in situ* phenotype, and the authentic *in vivo* and/or *in situ* phenotype can be the result of a physiological process, a pathological process, a cell and/or tissue development and differentiation, or a drug response, or a naturally occurring degradation process. The pathological process can for example be an inflammation, a secondary tumor deposit, or tumor vasculature. The authentic *in vivo* and/or *in situ* phenotype can also be represented by suspended cells from a tissue or body fluid, or such cells pelleted.

20 The displayed target structure can also be a molecule released from cells, e.g. tumor cells. Such a molecule can be released actively as well as passively.

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The displayed target structure can also be located in a cell surface, e.g. a cell membrane, as well as intracellularly or extracellularly of a cell surface. It can also be located intranuclear of a nuclear membrane. Examples of cell associated displayed target structures are different sets of epitopes, ligands, receptors, adhesion molecules, matrix molecules or matrix associated molecules. The displayed target structure can be based on protein, carbohydrate, nucleic acid, or lipid.

Sub C6
The displayed target structure can be obtained within a set of desired as well as undesired displayed target structures. In either case bound structures or unbound structures are recovered in dependence of the selection intended. A selection system can thus be a combination of a tissue phenotype subtractive approach, i.e. the use of both positive and negative selection, with the use of different types of libraries, e.g. large naive or semi-synthetic libraries.

The target structure displayed *in vivo* and/or *in situ* and representing authentic *in vivo* and/or *in situ* phenotype is preferably obtained from tissue sections by a histological technique which comprises freezing and/or fixation, and sectioning of a tissue sample. Such sections from a frozen tissue sample closely represent the original phenotype of all components at the moment the sample was frozen.

In practise a tissue is frozen *in vivo* immediately after surgical removal from a human being or an animal and the displayed target structure is localized *in situ*. For the skilled man there is no principal difference between a frozen tissue section and the same tissue *in vivo*.

The tissue sections can be pre-treated with enzyme or by chemical means, the enzyme pre-treatment being performed with a protease and/or a polysaccharase, and/or a ribonuclease, and/or a nuclease.

However, the authentic *in vivo* and/or *in situ* phenotype can also be obtained from body fluids. The body fluids can for example be blood, suspension of bone marrow, lymph, sperm, cerebrospinal fluid, or secretions

from cells. The secretions can be secreted actively as well as passively.
Actively secreted secretions are for example cytokines.

The first library of binding structure is according to the invention can be a naive, synthetic, or semi-synthetic antibody library. It can also be a combinatorial and/or preselected library, the combinatorial and/or preselected library preferably being a library produced by immunization against one or more displayed target structures. However, the combinatorial and/or preselected library can also be a chemical library.

According to the invention a first library of one or several binding structures is linked to genetic and/or other identifying information. Preferably, the linkage between such binding structures and genetic and/or other identifying information comprises particles of a filamentous phage or of any other virus. The linkage can also comprise polysomes or coded beads, i.e. beads identified by means of coding.

Bound binding structures are recovered by means of cleavage on condition that the cleavage site maintains the amplification ability. Preferably, the cleavage site is between the binding structure and a phage protein. Bound binding structures can also be recovered by disruption of the interaction between binding structure and target structure by chemical denaturation, e.g. chemically based elution such as treatment with an acid or alkaline solution, e.g. triethylamine.

Pasqualini and Ruoslathi (Nature 380: 364-366, 1996) discloses a technique for selection of phage particles. Anyhow, this technique is clearly distinguished from the present invention in that it is profoundly restricted to selection towards the intravascularly accessible endothelial cell surface.

EXAMPLES

Unless stated otherwise, tumors used for model experiments originated from the human colon adenocarcinoma cell line Colo 205 (American Type Culture Collection, ATCC, Rockville, MD), after subcutaneous growth in SCID (severe combined immunodeficiency) mice (Bommice, obtained from Bomholtgaard, Ry, Denmark). Human tumor and normal tissue biopsies and

Sub C₉ human peripheral blood samples were obtained from Lund University Hospital and Malmö General Hospital, Sweden. Human melanoma cell lines FM3 (kind gift from Dr. Jesper Zeuthen) and FMEX (ATCC) were used in FACS analyses. All tissues were snap-frozen in isopentane cooled in liquid nitrogen and stored at 70°C until cryostat sectioned. Six µm frozen sections 3×4 mm wide were mounted on slides and air dried overnight (o/n).

Vectors and scFv libraries.

Sub C₁₀ The different phagemid vectors, all based on pBR322, were equipped with ampicillin or chloramphenicol resistance genes and a gene for the scFv-M13 pIII (residues 249-406) fusion protein expressed from the *lac* or the *phoA* promoter with the secretion directed by either the *ompA* or the ST II signal peptide. An amber stop codon allowed for production of soluble scFv molecules in non-suppressor strains. The fusion proteins contained either a recognition site for His64Ala subtilisin (Ala-Ala-His-Tyr) (17) or Restriction Protease Factor Xa (Ile-Glu-Gly-Arg) situated amino terminal of pIII. An expression plasmid vector carrying a kanamycin resistance gene and the *lac* promoter was constructed for cassette insertion of scFv fragments in frame fusion with the superantigen staphylococcal enterotoxin A (SEA). The model scFv antibody constructs were derived from the C215 antibody directed to a well characterized epithelial antigen (the affinity for Fab binding is 2.3 nM) (24), and from the D1.3 anti-lysozyme antibody (25). For construction of a scFv antibody library first strand cDNA was synthesized from total mRNA from lymph nodes of a Cynomolgus Macaque immunized with a suspension of pooled human malignant melanoma metastases mixed with alum adjuvant. Family specific sense primers annealing to the first framework region of human VH (IgG) and VL (lambda, only) genes and antisense primers annealing in the CH1 and CL regions respectively were used for the first PCR. ScFv genes, VL-(Gly4Ser)₃-VH, were assembled and inserted into MluI and XhoI sites of the phagemid vector. The phagemid pool was transformed to *E. coli* TG-1 cells. Three times 10⁷ primary transformants were spread on minimal-agar plates, grown and pooled before superinfected with M13K07

helper phage (Promega). Phage rescue and culture of phage libraries and model phage constructs were according to standard techniques (5,6,26).

Selection of tissue binding phage.

5 ~~LC11~~ Tissue sections were air-dried on slides, fixed in acetone at -20°C for 10 min and rehydrated in 20% fetal calf serum (FCS) in TBS in a humid atmosphere for 1 hour at room temperature. Model or library antibody phage in 100 ml 20% FCS were incubated at 4°C o/n. The slides were washed 6×10 minutes by gentle agitation in 40 ml TBS in 50 ml Falcon tubes. Depending on the elution method, either of the following steps were performed: (i) Washes for 2×5 min in 50 mM Tris pH 7.6/1 M NaCl and 2 times 10 in 1×PBS pH 7.6. Phage were eluted with 300 µl 0.1 M triethylamine for 15 min and neutralized with 150µl 1M Tris pH 7.4. (ii) Washes for 2×5 min in 1 M NaCl, 10 mM Tris-HCl, 6 mM CaCl₂, 1 mM EDTA, pH 8.0 (Ala-64 subtilisin buffer) or 100 mM NaCl, 50 mM Tris-HCl, 1 mM CaCl₂, pH 8.0 (restriction 15 factor Xa buffer). Bound phage particles were eluted by volume 300 µl 30 mg/ml mutant Ala64-subtilisin for 30 min or volume 300 µl 100 mg/ml restriction factor Xa (New England Biolabs, Beverly, MA) for 2 h. All washing and elution steps were performed at room temperature. Phage titres of incubation solutions and eluates were determined by counting antibiotic 20 resistant colony forming units (CFU).

Soluble antibodies.

Single colonies of phagemid infected HB2151 *E. coli* were transferred to 96 Micro well plates (Nunc, Denmark) and grown at 37°C in 2×YT supplemented with cm or amp and 2% glucose for 17 h. Aliquots were 25 transferred to plates with fresh media with antibiotics, either 2×YT without glucose (*lac* promoter) or to a defined medium (27) (*phoA* promoter) and cultured at 30°C for 17 hours. The Micro well plates were centrifuged and the supernatants were transferred to new plates with an equal volume/well of 1% BSA. Similarly scFv-SEA fusion proteins were produced by culturing 30 transformed *E. coli* UL635 at 30°C or lower. The integrity of the fusion protein was confirmed by Western blot analysis using 5 µg/ml biotinylated

rabbit anti-SEA Ig. The fusion proteins were quantified in a sandwich type ELISA using rabbit anti-SEA antibodies as capture and biotinylated anti-SEA Ig as detector antibodies (28).

Immunohistochemistry.

5 ~~Dr C.12~~ Tissue sections first treated as described above were blocked with avidin 15 min and then with biotin 15 min diluted 1/6. Primary antibodies, scFv or scFv-SEA were incubated for 1 h, followed by secondary 1 mg/ml affinity purified rabbit antibodies to the ATPAKSE tag peptide or 5 mg/ml rabbit antibodies to SEA for 30 min, and biotinylated goat anti-rabbit Mab
10 diluted 1/1000 (Sigma) for 30 min, StreptABComplex HRP (DAKO) diluted 1/110 in 50 mM Tris for 30 min. Between all steps the sections were washed 3 times in TBS. Antibodies, avidin and biotin were diluted in 20% FCS in TBS. The staining reaction was developed for 8 min in 0.5 mg/ml DAB (3,3'-diaminobenzidine tetrahydrochloride, Sigma) dissolved in Tris pH 7.6 with
15 0.01 percent H₂O₂. The slides were rinsed 10 min in tap water and gradually dehydrated in 70-95 % ethanol and Xylene before mounting in DPX medium (Sigma).

Flow cytometry.

~~Dr C.13~~ FM3 or FMEX melanoma cells, 200 000 CELLS in 100 ml 1% BSA per
20 tube, were incubated on ice 1h with the primary antibody scFv K373-SEA (no primary antibody for the negative control), 30 min with 1 mg/ml rabbit anti-SEA Ig and 30 min with fluorescein-conjugated donkey anti-rabbit Ig (Amersham Life Science) diluted 1/100. Two washes in 1% BSA followed each step. The samples were analyzed by FACS (Becton Dickinson).

25 The colon epithelial tumor specific C215 and the unspecific control D1.3 scFv phagemid constructs were used to develop and evaluate conditions for phage binding washing, and elution. To confirm enrichment capabilities in an unrelated antigen system, the K378 scFv clone selected from a melanoma immune library was included in some experiments.

Example 1.**Optimal phage concentration.**

Dilutions of a 15:1 mixture of phage displaying scFv D1.3 (7.4×10^{11}) chloramphenicol resistant colony forming units, CFUcm) and scFv C215 (5.1×10^{10} ampicillin resistant CFU, CFUamp) were added to slides holding two 3 x 4 mm sections of Colo 205 tumor tissue. Non-specific binding of phage (D1.3) remained low (about 10^{-7} of input) when total number of phage added (in 100 μ l) exceeded 10^9 . Below that level, non-specific yield increased more than 10 X, reducing enrichment from over 1000 X to less than 50 X. In contrast the yield of antigen specific C215 phage remained equally high (10^{-4}) in the range of concentrations tested, demonstrating that more than 5×10^6 C215 epitopes were available for phage binding under these conditions. (FIG 1).

Example 2.**Antigen specificity of phage enrichment.**

To demonstrate antigen specificity of phage interaction with the tissue surface, C215 epitopes in sections of colon carcinoma tissue were blocked with an excess of C215 Mab prior to incubation with scFv C215 or scFv D1.3 displaying phage. The yield of specific phage was reduced to the level of the control phage for tissue antigens blocked with Mab, while the yield of D1.3 phage remained unchanged (FIG 5). Specificity was further demonstrated using crude concentrated culture supernatants containing soluble scFv C215 or the melanoma reactive scFv K378 antibodies that specifically could inhibit enrichment of the corresponding antigen-specific phage (not shown).

Example 3.**Proteolytic elution of phage.**

Proteolytic cleavage sites between the scFv antibody and phage minor coat protein (pIII) were introduced to evaluate the efficiency of proteolytic elution. The yield of specific phage, C215 or K378, from homogenous antigen-positive tissues was consistently $1-5 \times 10^{-4}$; no significant

difference between the three elution methods, triethylamine, Ala64-subtilisin (17) and blood clotting Factor Xa was demonstrated. In contrast, yield of non-specific phage was always lower with the proteolytic elution methods, by a factor of 1.5 to 4 thus improving enrichment to the same extent (Table 1).

Table 1.

Proteolytic and triethylamine elution of antibody phage from tissue sections.

10

Elution method	Yield of tumor-reactive phage (out/in \pm (STD, n=3)) $\times 10^{-1}$	Specificity	Yield of control (out/in \pm (STD, n=3)) $\times 10^{-1}$	Enrichment
15 A. Triethylamine	1,0 \pm 0,2	C215	530 \pm 340	19
Triethylamine				
C215 blocked**	0,028 \pm 0,002	"	380 \pm 89	1
B. Triethylamine	3,1 \pm 0,5	"	130 \pm 27	238
20 Ala64-subtilisin	1,4 \pm 0,6	"	33 \pm 24	424
C. Triethylamine	3,3*	"	30*	143
Restriction				
Factor Xa	2,9*	"	60*	483
25 D. Triethylamine	1,3 \pm 0,004	K378	9,1 \pm 2,0	1429
Ala64-subtilisin	1,5 \pm 0,4	"	6,1 \pm 0,8	2459

* Mean of duplicates

30 ** 20 μ g/ml C215 Mab

Example 4.

Successful enrichment depends on the relative amount of antigen positive surface area.

Sub C.14 Most antigens in a tissue section could be expected to distribute over only a fraction of the total tissue surface area (subpopulations of cells/substructures of tissue). As varying antigen distribution in individual tissue sections was difficult to reconstruct for model experiments, a mosaic of antigen negative and antigen positive sections was constructed. Slides covered with 30 tissue sections of Colo205 SCID tumor and human spleen in various proportions were produced. A total amount of 3.6×10^8 scFv C215 phage and 3.0×10^{10} control phage was added per slide. The yield of specific phage increased linearly with increasing number of antigen positive tissue sections, whilst the yield of scFv D1.3 phage was not effected (fluctuated two-fold or less) by the different proportions of tissues within the experiment (FIG 2). When only antigen-negative (spleen) tissue was applied, the yield of phage of the two populations was similar. Thus, no difference in intrinsic non-specific binding between the phage stocks was seen. Unspecific binding of D1.3 phage did not differ significantly (less than two-fold) between other non-antigen expressing tissue, chosen to represent lipid-rich, epithelial parenchymal and mesenchymal organs (human brain, C. Macaque liver, human spleen and human heart, respectively (data not shown). The effect on enrichment of specific phage by reducing the antigen-expressing surface fraction was also exemplified by a four-fold decrease in yield of specific C215 phage after binding to a primary colorectal cancer biopsy containing epithelial tumor cell areas together with smooth musculature layers and connective tissue of the normal colon components (not shown).

Example 5.

Efficiency of multiple rounds of selection.

Sub C.15 The first selection round aimed at rescue and enrichment of rare specific phage in a large library is critically dependent on both the specificity of the procedure, i.e. the enrichment factor and the capacity scale of the

system, i.e. the yield of enriched phage populations after selection. In the single pass experiments described, more than 10^4 phage were rescued after each round of selection, which when assuming an average enrichment factor of 100 times allows enrichment of specificities with original frequencies of one per million in the library. To demonstrate that this could be achieved in the tissue based selection system, four selection rounds were performed with the two model phage (C215:D1.3 phage) mixed in the following proportions, 1:100, 1:10⁴ and 1:10⁶. An increased yield of phage indicated enrichment of specific phage had occurred. After various selection rounds the ratio of specific to unspecific phage was determined by immunohistochemical staining of tissue sections with scFv antibodies produced from randomly picked single clones. From an original frequency of 1:10⁶ in the mixture, 89/95 (94%) clones stained sections with C215 specificity after three selection rounds. This represents a 98-fold enrichment in each pass (FIG 3A). From the 1:10⁴ mixture, two rounds of selection resulted in a similar enrichment level (average enrichment 93-fold).

Example 6.

Library selection in melanoma tissue sections.

To demonstrate that the tissue-based selection method could be used to identify scFv antibodies towards tissue expressed antigens in a random combinatorial library, a phage library derived from melanoma immunized non-human primates was selected on frozen sections of metastatic melanoma. In each selection round, melanoma tissue from different patients was used to promote enrichment of antibodies to epitopes common to this tumor type and select against antibodies to unique or allotypic markers. After three selection rounds, the yield of phage increased from 10^{-7} to 10^{-4} (FIG 3B). Ten clones from the second and third round of selection were randomly picked for PCR and restriction cleavage analysis. From the second round, 3/10 clones and from the third, 10/10 clones had a scFv antibody insert of correct size. *HinfI* and *RsaI* restriction patterns of these inserts revealed that all three clones in the second round, designated K373, K378

del C. 16 and K382, were unique and that all but one clone in the third round had a pattern identical to K378. The predominant representation of the K378 clone in the third round paralleled the increased phage yield at this stage. In a model experiment the scFv K378 phage was enriched 2490, 575 and 2 times over D1.3 in sections of two different melanoma samples and of human spleen (not shown). The scFv antibody genes of K373 and K378 were re-cloned and expressed as fusion proteins with staphylococcal enterotoxin A (SEA) which was used as detection "tag" in immunoassays.

Example 7.

10 **The scFv K373-SEA fusion protein binds to intact melanoma cells and tissue sections.**

del C. 17 The scFv K373-SEA fusion protein was demonstrated by FACS analyses to bind to cultured cells of the FM3 (FIG 4A) and FMEX (not shown) melanomas but not to human peripheral blood lymphocytes (FIG 4B). The intensity of the staining indicated that the epitope was strongly and homogeneously expressed on viable cells and thus demonstrated the cloning of a cell surface reactive antibody phage through the use of a tissue-based selection method. The scFv K373-SEA but not the scFv D1.3-SEA fusion protein strongly and homogeneously stained melanoma cells in sections of metastatic melanoma tissue (not shown). By FACS analyses the K378-SEA fusion protein was found to bind only weakly to FM3 and not to FMEX or PBL. However, in tissue sections of metastatic melanoma this antibody bound strongly to both the melanoma cells and stroma components (not shown).

Example 8.

25 **Subtractive epitope phenotype selection.**

del C. 18 A mixture of filamentous phage particles displaying the C215 scFv (3.6×10^9 particles) specific for an antigen expressed in all human epithelia, including small intestine and large intestine (colon) and the 1F scFv displaying phage (1.1×10^8), reactive with only colonic epithelium, was applied to frozen sections of small intestine or control (uterus) tissue in order to perform a negative selection step. After overnight incubation at +4 C unbound

11 C 19) Phages in the supernatant were transferred to sections of colon epithelium in order to perform a positive selection step. After overnight incubation at +4 C sections were washed six times for 10 min with 50 mM TRIS, pH 7.6 with 0.15 M NaCl. By incubation with Ala64-subtilisin (33 µg/ml) at RT bound particles were eluted and used to infect *E. coli* DH5alphaF^r bacterial cells. Infected bacterial cells were grown as single colonies on agar plates. colonies were counted and used to estimate the number of phage particles in the eluates. By using different antibiotic resistance genes for the two scFv phagemid constructs the number of phage type could be estimated.

10 The results (FIG 6) demonstrate that by negative selection on the crossreactive tissue (small intestine) the number of phage particles of the crossreactive type (C215) could be reduced about 4 times as compared to negative selection on control tissue, while no reduction was recorded for the colon specific phage antibody particles.

15 By negative selection the frequency of specific phage particles could be enriched 4 times in a single pass phage enrichment experiment as compared to crossreactive phage antibodies. Similarly, phage antibodies can be obtained which define an epitope unique to one tissue and not present in another. Thus, as on small intestine in this case a negative selection specifically removes undesired crossreactive phage antibodies to promote enrichment of phenotype specific specificities.

11 C 20) According to the method of the invention antibody phage can be directly selected in frozen tissue sections. The concentration of phage applied to the tissue sections influenced non-specific binding and efficiency of positive enrichment. Concentrations higher than $5 \times 10^9 / 100 \mu\text{l}$ reduced yield of non-specific phage, indicating that a limited number of high-affinity non-specific binding sites could be saturated at these concentrations. This was in contrast to low-affinity non-saturable sites responsible for a relatively constant yield (about 10^{-6} of input) of unspecifically bound phage particles within the high concentration range. Saturation of specific C215 epitopes could not be achieved even at the highest practicable phage con-

concentration, even though only a fraction ($<5 \times 10^6$) of the estimated number of available tissue epitopes ($>10^{10}$) were utilized for phage binding. Phages displaying antibody fragments (about 25 percent of the scFv C215 population by Western blot analysis, not shown) were present in large excess (>2000 -fold) as compared to bound and eluted phage. At the highest phage concentration used, corresponding to ≈ 0.2 nM of C215 displaying phage, >6 percent of soluble antibody (affinity 2.3 nM) would be bound at equilibrium. This discrepancy suggests that other factors, e.g. steric hindrance and diffusion limitations will be important to consider for further improvement of binding efficiency and selection capacity.

Proteolytic elution was shown to reduce retrieval of non-specifically bound phage and thus improved enrichment by adding antigen selectivity to the elution process. It will also elute specific phage irrespective of the nature and strength of the antigen-antibody interaction, thereby making the technique more generally applicable.

In single-pass experiments, enrichment of a few hundred times was routinely obtained, as compared to the relatively low enrichment factors (19 at the low end, Table 1) seen before optimization. After optimization a useful enrichment of antibodies could be achieved even using heterogenous samples with only 3 percent of antigen-specific surface area, mimicking cell subpopulations/substructures of a tissue (FIG 2). The million-fold enrichment achieved in three selection rounds on tissue sections was comparable to phage selection using cell suspensions (8,12) and to use of cultured epithelial cells for enrichment of the same scFv C215 phage (not shown) and to pure antigen systems (1). For a given enrichment factor, the probability of rescuing rare specific phage will directly correlate to the number of eluted phage (which is limited by the total capacity of the selection system); this suggests that the high-end of the concentration range investigated (10^{10} - 10^{11} CFU per 100 μ l), should optimally be used.

The method according to the invention demonstrates that sections of frozen tissue can be used for "panning" of antibody phage libraries. The

specificity of the phage particle interaction with tissue-expressed epitopes was demonstrated by the complete lack of specific enrichment in epitope-blocked and antigen-negative sections, including antigen negative samples of tissues of various histogenetic origin and biochemical composition.

5 Blocking ("masking") of epitopes in a tissue with Mabs or Fab/scFv antibody fragments of already identified specificities may be applied to promote enrichment of novel and less abundant specificities in a library in order to gradually dissect different epitopes of a tissue as shown previously in pure antigen systems (18). By selection of a phage library derived from a human
10 melanoma-immunized primate using metastatic melanoma tissue sections, a clone was identified which stained melanoma cells in tissue sections and defined a cell surface antigen expressed in cultured human melanoma cells but not in human peripheral blood mononuclear cells. The specificity of this cloned antibody (K373) will be further investigated by extended immuno-
15 histochemical analysis.

~~Jul 23~~ Mabs to melanoma-associated antigens resulting from immunization of non-human primates have previously not been established, although primates have been used previously for tumor-immunization to produce polyclonal antibody reagents (19-21). Moreover, weak humoral and cellular
20 immune responses to the autologous tumor in melanoma patients have clearly demonstrated the presence of melanoma antigens which are immunogenic to humans (22). By the choice of a primate rather than a rodent for immunization with human material, broad antibody responses to various normal human tissue components can be avoided (19). This allows
25 the generation of a greater variety of more discriminating antibody specificities e.g. to tumor-associated antigens. However, any immunization procedure will be biased by the immune repertoire of the species used.

The present invention describes the development and application of a highly efficient method for the direct selection of a binding structure as an
30 antibody phage towards displayed target structures expressed *in vivo* and represented by antigens *in situ* in cryostat tissue sections. In a model system,

scFv phage directed towards an epitope on the GA733-2 epithelial glycoprotein expressed in colorectal carcinoma tissue could be specifically enriched by up to 1500 times in single-pass experiments. Enrichment efficiency was directly proportional to the fraction of antigen positive sections over the total surface area. Sufficient enrichment was achieved at a fraction less than four percent, allowing for the selection of antibodies to subpopulations of cells or to tissue substructures. Successful selection of metastatic melanoma cell surface and cell surface/matrix binding clones from a combinatorial scFv antibody phage library derived from melanoma immunized non-human primates, demonstrated the applicability of the method.

The present invention is applicable not only to human and animal tissues but also to all other living organisms, e.g. plants, fungi, prokaryotes, and other non-mammalian organisms.

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